Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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To cite this Article Scovell, William M.(1989) 'The Structural and Possible Functional Alterations on DNA and Chromatin Resulting from *cis*-$Pt(NH_3)_2Cl_2$ Modification', Journal of Macromolecular Science, Part A, 26: 2, 455 — 480
To link to this Article: DOI: 10.1080/00222338908051987
URL: http://dx.doi.org/10.1080/00222338908051987

J. MACROMOL. SCI.–CHEM., A26(2 & 3), pp. 455-480 (1989)

THE STRUCTURAL AND POSSIBLE FUNCTIONAL ALTERATIONS ON DNA AND CHROMATIN RESULTING FROM *cis*-$Pt(NH_3)_2Cl_2$ MODIFICATION

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ABSTRACT

cis-Diamminedichloroplatinum(II) binds covalently to the bases of DNA and exhibits a number of distinct modes of binding that can influence the structure of DNA. Of these, the intrastrand crosslink to adjacent guanines appears responsible for unwinding of supercoiled DNA and stimulating S1 nuclease activity. Investigation of the binding that occurs in chromatin reveals that the antitumor drug also forms crosslinks between DNA and the HMG proteins 1, 2, and E in micrococcal nuclease-accessible regions, in addition to protein-protein crosslinks between the LMG proteins. From the studies on both DNA and chromatin, we propose 1) a model for the interaction of, and the general location of, these HMG proteins in chromatin and 2) novel mechanisms for the possible action of *cis*-diamminedichloroplatinum(II) in cancer chemotherapy.

INTRODUCTION

cis-$Pt(NH_3)_2Cl_2$ (DDP) is one of the most widely used drugs in the treatment of cancer today. Although effective against a number of forms of cancer, *cis*-DDP, in combination with other drugs, exhibits a cure rate of greater than 80% in the treatment of testicular cancers [1]. Notwithstanding its im-

portance in cancer chemotherapy, we are just beginning to define the types of interactions that can occur in the cell and relate these to possible alterations in biological functions. In addition to its role in halting cell division and therefore terminating the proliferation of cancerous cells, this drug may also influence other biological processes. It is with this in mind that I would like to address the subject.

My overall aim will be first to present some fundamental aspects of the interaction of DDP with DNA which are generally agreed upon and review what they may mean from a biological sense. Second, and I believe most important, I shall focus on some of our recent findings on DDP interacting with chromatin in which we have found that DDP covalently crosslinks some most intriguing proteins to DNA and we propose that these crosslinks may have an enormous impact on vital processes within the cell.

Figure 1 shows the structures of the *cis* and *trans* isomers of DDP. These complexes are similar in that they are four-coordinate, square planar, d^8 Pt(II) species, in which the ligands are oriented 90° with respect to each other. The Pt–N bonds are kinetically inert, while the Pt–Cl bonds are labile [2]. The most prominent chemical difference between the two isomers is that *cis*-DDP may engage in chelate formation with ligands, while the *trans*-DDP cannot. The renewal of interest in these complexes since about 1970, however, rests almost entirely on the finding that the *cis*-DDP isomer is effective in the treatment of cancer, while the *trans*-DDP is ineffective.

An early finding which highlighted this and perhaps foretold of the different therapeutic values in the two isomers indicated that the *cis* isomer was strikingly more effective than the *trans*-DDP in reducing the survival of HeLa

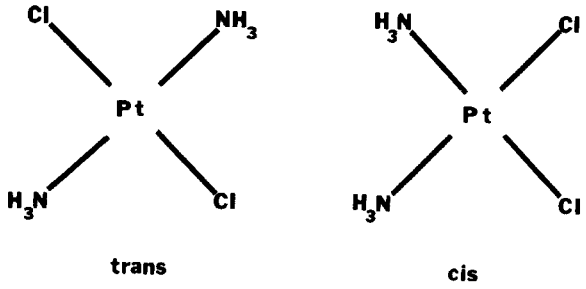


FIG. 1. The structure of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$.

Ligand Substitution Reactions

a. Aquation (pH dependent)

$$(NH_3)_2PtCl_2 \overset{H_2O}{\rightleftharpoons} (NH_3)_2Pt(Cl)(H_2O)^+ \overset{H_2O}{\rightleftharpoons} (NH_3)_2Pt(OH_2)_2^{+2}$$

b. General Ligation

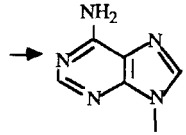
$$(NH_3)_2PtCl(H_2O)^+ + L \rightleftharpoons (NH_3)_2PtClL + H_2O$$

$$(NH_3)_2PtClL + L' \rightleftharpoons (NH_3)_2PtLL' + Cl^-$$

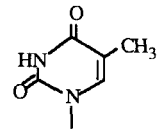
FIG. 2. The ligand substitution reactions for $Pt(NH_3)_2Cl_2$.

cells in culture [3]. In addition, it was found by a number of laboratories that *cis*-DDP selectively inhibited DNA synthesis in mammalian cells, both *in vitro* and *in vivo* [4-6].

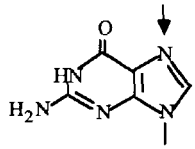
The reactions of the platinum(II) complexes take place via a substitution mechanism [2] at the Pt–Cl bonds, as shown in Fig. 2. Since the DDP complexes have two such bonds, they can form a monodentate linkage with one or two different ligands, or alternatively, form a chelate with a more complex ligand. With the previous findings in mind and since DNA may be regarded as the single most important macromolecule to the life of the cell, it has received the most attention in terms of DDP binding. The targets on DNA, shown in Fig. 3, are the nucleophilic nitrogens on the heterocyclic bases, adenine, thymine, guanine, and cytosine. The primary sites at which the *cis*-DDP binds to the nucleosides are at the N-1 on adenine, N-7 on guanine, and the N-1 site on cytosine, with the binding constants for the 1:1 complexes formed being about 10^4 [7-9]. Under comparable conditions, there is no reaction on thymine. If these findings can be extrapolated to double-helical DNA, one can predict [7] that the DDP binding will be more selective because the primary sites of interaction on adenine and cytosine are involved in complementary hydrogen bonding in the interior of the helix and therefore, inaccessible to the DDP. Therefore, the primary site of binding will be the N-7 position of guanine, which resides in the major groove of B-form DNA. To be sure, DDP can also be expected to react with proteins and RNA, but the effect on cell proliferation would not be expected to be as important. Figure 4 indicates the nucleophilic sites which are the most likely candidates in both the DNA and protein molecules.



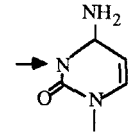
Adenine (A)



Thymine (T)



Guanine (G)



Cytosine (C)

FIG. 3. The structures of the bases in DNA and the primary sites for $Pt(NH_3)_2Cl_2$ interaction.

BIOLOGICAL MACROMOLECULES AND POTENTIALLY REACTIVE SITES

I. Nucleic Acids	**Bases**	**Sites**
	Adenine	**N-1, (N-7, N-3)**
	Thymine	
	Guanine	**N-7, (N-7, 0-6)**
	Cytosine	**N-3**
II. Protein	**R Groups**	
	S containing	**Met, Cys**
	Acidic	**Glu, Asp**
	Basic	**His**

FIG. 4. The primary nucleophilic sites in DNA and proteins.

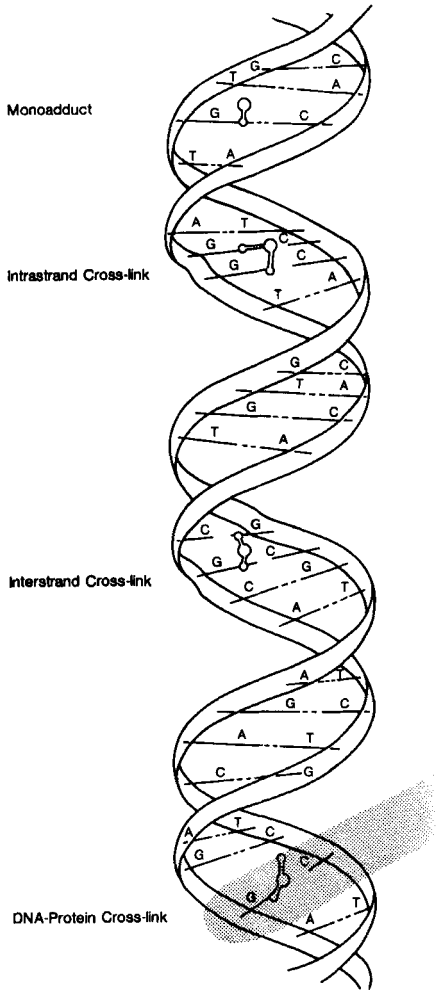


FIG. 5. A DNA model showing the primary modes of *cis*-$Pt(NH_3)_2Cl_2$ binding in DNA and in chromatin.

The predominant modes of binding that *cis*-DDP display on DNA are shown in Fig. 5. These involve monodentate binding to guanine, an interstrand crosslink or an intrastrand crosslinks between two adjacent guanines

on the same strand. An intrastrand crosslink involving nonadjacent Gs and/or A nucleotides of the type GNG and ANG has also been reported [10]. In addition, when the DNA is in tight association with proteins as is the case in the nucleus of the cell, the DDP can also form a crosslink between the DNA and protein if they are in close contact and reactive nucleophilic amino acid residues are positioned appropriately.

BINDING OF *cis*- AND *trans*-DDP TO SUPERCOILED SV40 DNA

Both the *cis*- and the *trans*-DDP can bind to DNA, but they produce different effects. One of the first differences reported was that the binding of the *cis*-DDP isomer to supercoiled, SV40 DNA produced a much larger unwinding angle than that of the *trans*-DDP. In our first report, it was shown to exhibit, on average, twice the unwinding angle as that for the *trans*-DDP [9]. In addition, it appeared that it exerted a larger effect during the initial binding of the first few DDPs. We suggested then that the larger unwinding angle of the *cis*-DDP was due to it forming an intrastrand crosslink to adjacent guanines and producing a "*cis*-DDP pinch" in the DNA backbone, which effectively unwinds the DNA. The effect was most pronounced at low levels of binding, in part, because this is the point at which it was most probable to exhibit this binding mode. The *trans* isomer, of course, is stereochemically disallowed from exhibiting this mode of binding and produces a small alteration in the DNA structure [11]. Additional evidence for this intrastrand mode of binding for *cis*-DDP came from studies with nucleases on the modified DNA [12-14]. It was found that *cis*-DDP binding on DNA stimulated far more S1 nuclease activity than did the *trans*-DDP. Since S1 nuclease is specific for single-stranded DNA, it was proposed that the intrastrand crosslinks formed by the *cis*-DDP distorted the DNA backbone and produced single-stranded regions which were recognized and cut out by S1 [13, 14]. In addition, this effect was most dramatic when the influence of *cis*-DDP binding on S1 activity was compared on poly(dG).-poly(dC), in which an intrastrand crosslink would predominate, and poly(dG-dC).poly(dG-dC), in which no such crosslink could occur [14]. *cis*-DDP was found to stimulate significant S1 activity on poly(dG).poly(dC) at all r_b levels (r_b = moles DDP bound/moles DNA(N)), while in the alternating copolymer, poly(dG-dC).poly(dG-dC), there was little stimulation over the same r_b range. On the other hand, the effect of *trans*-DDP binding on these two synthetic DNAs was comparable and small in both cases. At this early stage in this work, these were some of the first differences to be recognized in the character of the two isomers. Since then, other studies provided additional evidence supporting the presence of this form of the

intrastrand crosslink [15-17]. In addition, Eastman has determined that the platinum adduct involved in the intrastrand crosslink with adjacent guanines is the predominant mode of binding for *cis*-DDP on DNA at low levels of binding [18].

RELATIVE DISTRIBUTION OF *cis*- AND *trans*-DDP IN THE SV40 GENOME

In an attempt to determine if there exists a sequence or sequences in a biologically interesting DNA which *cis*-DDP may bind to preferentially, we extended our studies to the DNA from the SV40 virus [19]. The advantage of examining this DNA are many, including the fact that it is small genome (4326 bp), contains only five genes, is completely sequenced [20] and the information gained on the DNA may perhaps be used to predict the influence of *cis*-DDP on the SV40 minichromosome. In this vein it has been reported that *cis*-DDP inactivates the extracellular SV40 virus [21] and also inhibits SV40 DNA replication in African green monkey kidney cells [22].

The physical and genetic map [20] for SV40 DNA is shown in Fig. 6. The developmental cycle for the virus starts with the transcription of a large T and small t antigen, after which the translated large T antigen binds to specific sequences in the regulatory region (ori region) of the genome. The binding is essential for viral replication and for transcription of the coat proteins for the virus. The notations in the center of the circle indicate the unique sites at which a number of restriction endonucleases cleave the DNA. These include Bgl 1, Kpn I, Hpa II, Eco RI, and Bam HI. The last important point to be made about SV40 DNA is that although the DNA is 57% (A+T) rich, the regulatory region is very rich in its (G+C) content. This, therefore, might be expected to be a preferred site for *cis*-DDP binding. If *cis*-DDP exhibited this preference, and eliminated or made it difficult for sequence-specific regulatory proteins to bind in this region, which is essential for the initiation of viral replication or transcription, it would exert a profound effect on the life of the virus.

To examine the distribution of *cis*- and *trans*-DDP within or about specific locations in the genome, we monitored the relative cleavage inhibition of a number of (sequence-specific) restriction endonucleases [19]. The recognition sequence for each restriction enzyme, shown in Fig. 7, is different and contains a unique sequence and (G+C) composition. The analysis is based on the premise that if DDP binds at, or immediately adjacent to, the recognition site, it will disrupt the binding to, and the cutting of, the DNA at that

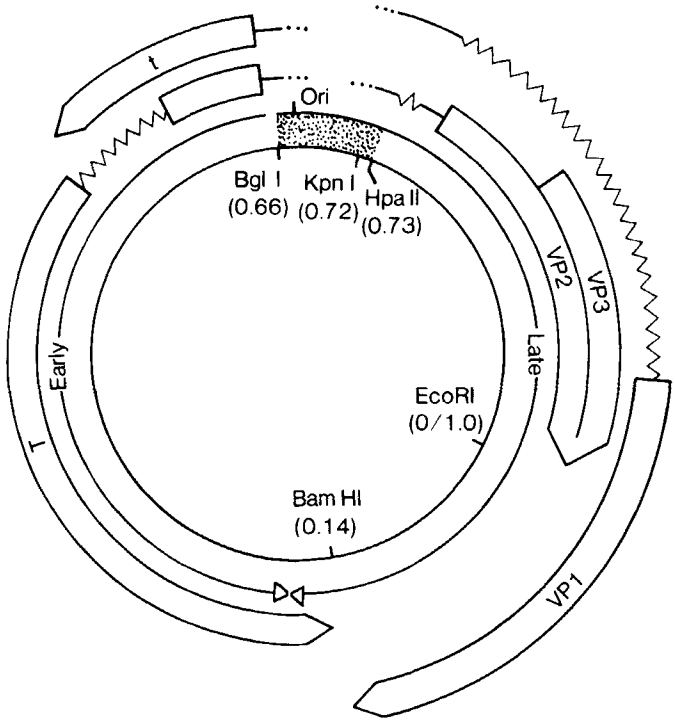


FIG. 6. Schematic representation of SV40 viral genome, indicating the locations of principal features. The genome is represented as a circle with the origin of replication (ori) at the top. Restriction endonuclease cutting sites are shown for Bgl I, Kpn I, Hpa II, Eco RI, and Bam HI. The genome is divided into coding sequences for the early and late genes. The arcs with arrow heads indicate the coding regions of *m*RNAs. The name of the protein coded by these sequences is labeled within the arcs. The stippled area contains the regulatory region of the genome.

site. To a first approximation, it is assumed that the extent of cleavage inhibition will be proportional to the level of DDP bound in that region.

Figure 8 shows the electrophoretic pattern obtained in the experiment. Lane 1 shows the positions of the supercoiled and nicked forms of DNA, while Lanes 2-6 show cleavage of the unmodified DNA with Bam HI, Eco RI, Bgl I, Hpa II, and Kpn I, respectively. Lanes 7-11 show the results of the cutting on the *cis*-DDP-modified DNA, while Lanes 12-16 contain the analogous results

FIG. 7. Recognition and cleavage sites for restriction endonucleases. The recognition sequence plus 10 base pairs on each side is shown for Bam HI, Bgl I, Eco RI, Hpa II, and Kpn I. The base pairs essential in the recognition sequence are underlined with a solid line. Note that the central five base pairs for Bgl I, underlined with a broken line, is not an essential sequence. Tracts of two or more guanines are shown shaded. The vertical lines within the recognition sequence designate the cutting sites.

for the *trans*-DDP-modified DNA. An analysis of the relative band intensities reveals that the inhibition of cleavage produced by both isomers follows the order: Bgl I $\gtrsim$ Bam HI > Hpa II, Kpn I > Eco RI. The inhibition is related to the content of guanines in and about the immediate vicinity of the recognition sequence, but not simply within the site. Tracts of guanines of two or more appear to have a disproportionate effect on the activity of the enzyme, which suggests that DDP binding may be enhanced by the sequence of guanines in the DNA, not simply the composition itself. Specifically, in terms of SV40 DNA sequences within the regulatory region (i.e., in the vicinity of the Bgl I site), they can be considered as *hyper-reactive* toward DDP binding.

PROPOSAL FOR THE INTERACTION OF *cis*-DDP WITH THE SV40 MINICHROMOSOME

The real entity of concern in the cell, however, is not the naked DNA, but is the chromosomal material [23]. In this nucleoprotein assembly, exhibiting

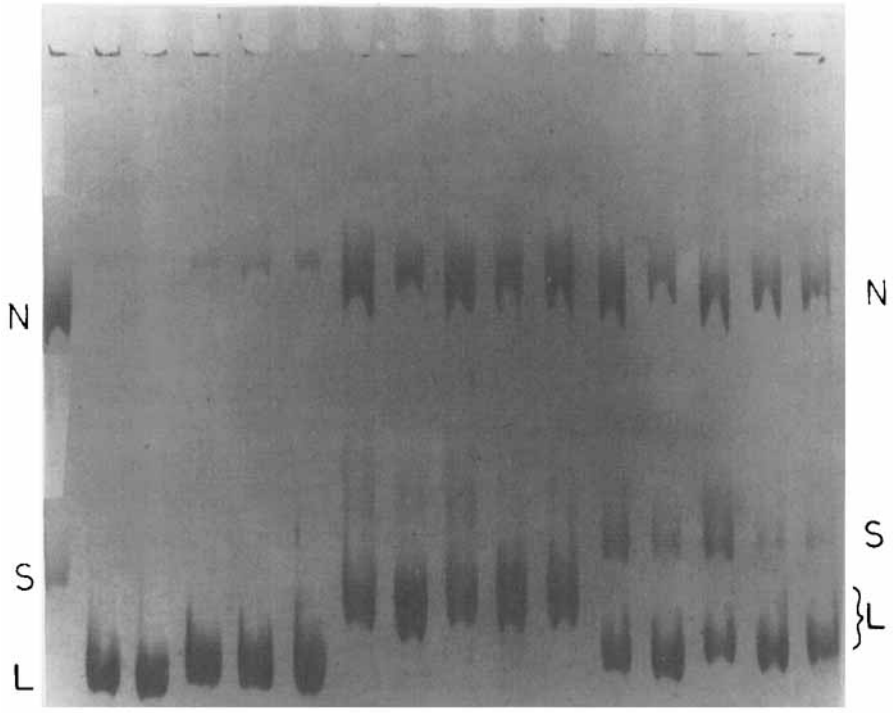


FIG. 8. Cleavage of *cis*- and *trans*-DDP modified-SV40 DNA by restriction endonucleases which cut at a single, unique site. Lane 1 shows the mobility of nicked (N) and supercoiled (S) forms of SV40 DNA. Lanes 2-6 contain SV40 DNA cleaved with Bam HI, Eco RI, Bgl 1, Hpa II, and Kpn I, respectively. These controls demonstrate complete cleavage to the linear (L) form. Lanes 7-11 contain SV40 DNA [9.0×10^{-5} *M* DNA(N)] which has reacted with *cis*-DDP (18 μmol/L) for 3 h at 25°C, spot dialyzed, and then cleaved with the restriction enzyme. The order is the same as in the control series. Lanes 12-16 contain SV40 DNA reacted similarly with *trans*-DDP followed by cleavage with restriction enzymes, again in the same order as the controls.

a higher-order structure, the DNA is complexed with a multitude of proteins many of which may shield or preferentially expose essential DNA sequences. In the case of the developing SV40 virus, this assembly is the SV40 mini-chromosome (MC), shown in the electron micrograph [24] in Fig. 9. The MC is composed of the basic nucleosome subunits just as observed in a

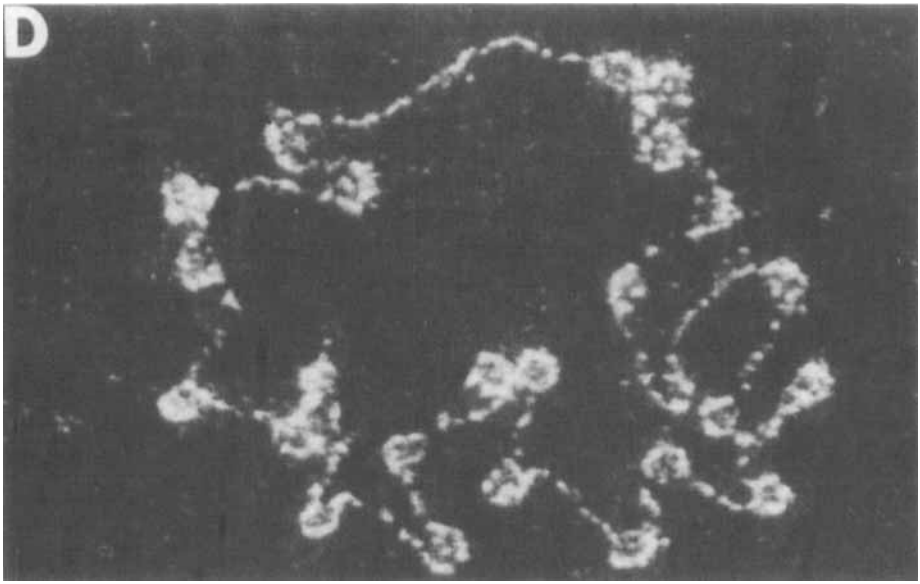


FIG. 9. Electron micrograph of an SV40 minichromosome, which shows a stretch of the genome devoid of nucleosomes. This gap has been mapped from the Bgl 1 site to be about 0.74 on the physical map. The nucleosomes appear as white circular clusters along with the circular DNA. The nucleosome-free region is at the top in the center.

eukaryote genome, but experimentally, it presents the advantage that it is much smaller, better characterized, and represents an excellent model system for the larger eukaryotic chromosome. Of particular interest here is that in a fraction of MCs, the nucleosomes appear to be, more or less, randomly arranged on the DNA, except for one particular region of about 400 base pairs [25-27]. This nucleosome-free region, shown at the top of Fig. 9, extends from about the Bgl I site to about the Hpa II site, and is considered "open" and topologically exposed as evidenced from nuclease digestion studies. One can reasonably extend our findings on the DNA level and suggest that the *cis*-DDP reactivity toward sequences in the regulatory region may be even greater in the MC since many of the other binding sites would be shielded to some extent by the close association with the core histones. Considering the findings that the sequences in the regulatory region of SV40 DNA are (G+C) rich and hyper-reactive to *cis*-DDP, it is quite possible that this may have a

direct bearing on the report that *cis*-DDP inhibits SV40 viral replication and the production of the late proteins [22]. The biological consequence of the *cis*-DDP binding to the MC may be profound, especially if it modulates or obstructs the binding of regulatory proteins in this region. The large T antigen, for example, binds in a sequence-specific manner and makes contacts with a number of guanines in the major groove. This interaction is considered to be an essential step during the initiation of DNA replication [28, 29]. *cis*-DDP modification of these same guanines may be expected to reduce the large T antigen binding to the regulatory sequences, to perhaps a degree greater than that observed from the methylation of the sites. This is a proposal which awaits experimental test.

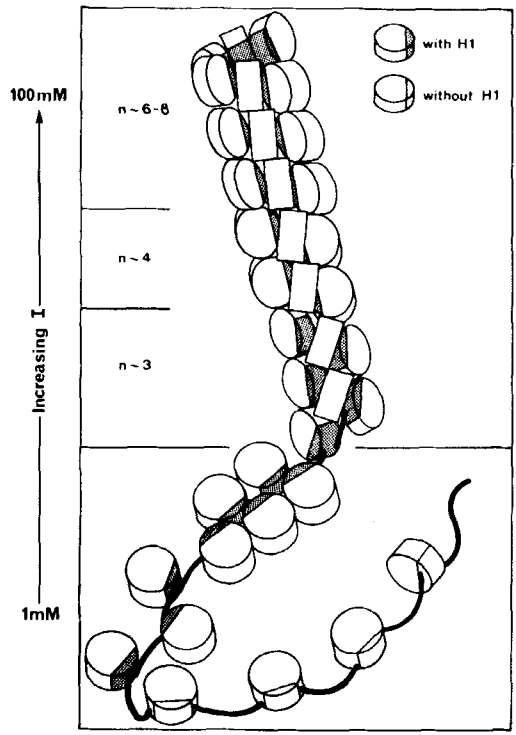


FIG. 10. Idealized drawing of helical superstructures formed by chromatin containing H1 with increasing ionic strength. The open zigzag of nucleosomes (bottom left) closes up to form helices with increasing numbers of nucleosomes per turn (n). When H1 is absent (pictured at bottom right), no zigzags or definite higher-order structures are found.

cis-DDP INTERACTION WITH CHROMATIN

Our lab has also expended a great deal of effort in studying the *cis*-DDP interaction with cellular chromatin. As with the SV40 investigations, we regard the findings on DNA as providing the basis for understanding the influence of *cis*-DDP within the cell, but consider this as only a model system to guide our thinking in the more complex chromatin within the cell nucleus. A simple schematic representation of chromatin is shown in Fig. 10, which reveals at the bottom, the extended chromatin structure, with individual nucleosomes having no H1 histone protein [30]; experiments indicate that chromatin has this "open" structure at very low salt concentration. As the salt concentration increases toward that of physiological conditions, one finds the nucleosomes associate with each other to produce a higher-order structure characterized by fibers of about 30 nm. It is germane to point out that in this current model of chromatin, only the histone proteins, the H1 protein and the core histones, H4, H3, H2a, and H2b, have been considered. None of the multitude of nonhistone chromosomal proteins is part of this working model.

We had two main objectives in the studies with chromatin. The first was to determine the influence of DDP binding on chromatin structure and to consider the implications of this toward the possible disruption of vital cellular functions. Second, *cis*-DDP forms covalent bonds and can form crosslinks between DNA and proteins which can be readily reversed by chemical means. Therefore, the drug may serve as a small, crosslinking agent which may be used as a unique probe to determine the relative location of these proteins within chromatin and perhaps their location with respect to particular gene sequences.

cis-DDP CROSSLINKS NONHISTONE CHROMOSOMAL PROTEINS (NHCP)

The first experiments dealt with monitoring the time-dependent effect of *cis*-DDP binding on the character of the nuclear proteins [31]. After reaction of the nuclei, aliquots were removed at increasing times and run on SDS-PAGE. Over a 5-h period, it was observed on the gel that the *cis*-DDP binding had little or no effect on the intensity of the core histones, but appeared to progressively reduce the level of NHCP. Due to the inherently weak intensities of these latter bands, the experiment was repeated, but this time the NHCP were extracted at each time point, precipitated, and then run in this more concentrated form on the gel. Figure 11 shows the control and *cis*-DDP-treated samples up to 5-h

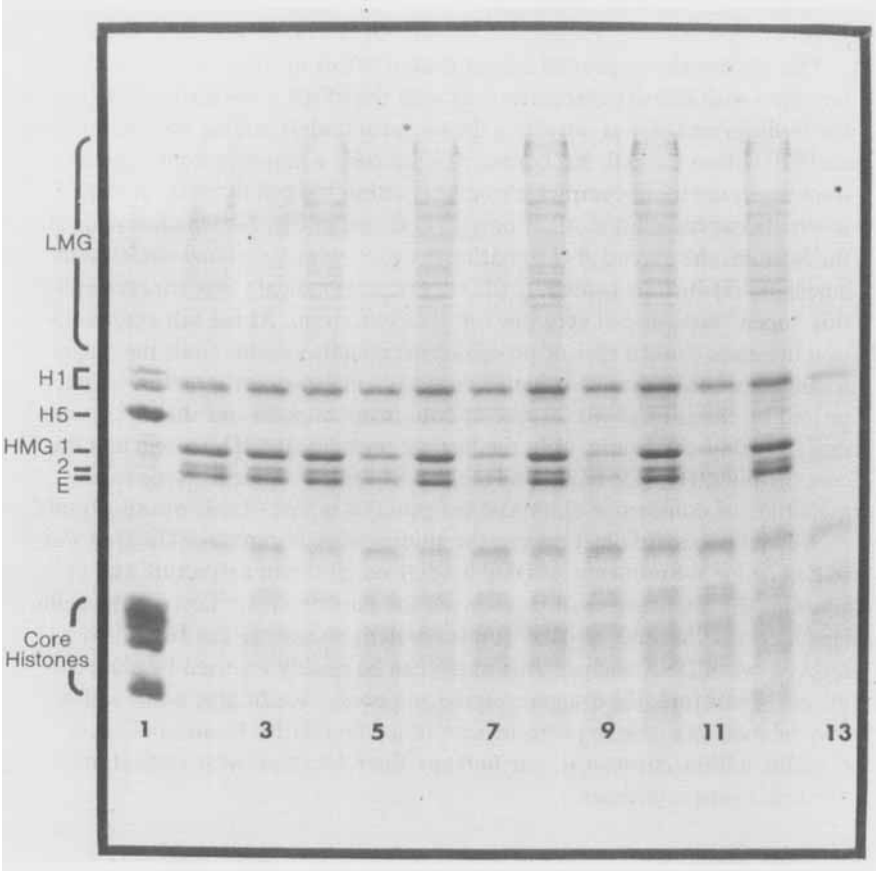


FIG. 11. SDS polyacrylamide gel electrophoresis of extracted nuclear proteins after *cis*-DDP reaction with chicken erythrocyte nuclei. *cis*-DDP was reacted with nuclei for 0, 30 min, 1, 2, 3, and 5 h at 25°C at $r_i = 0.25$ (r_i = moles DDP reacted/moles DNA nucleotide). Lane 1 displays proteins from unextracted nuclei control. Lanes 3 and the odd-numbered lanes contain the 0.35 *M* NaCl extract from nuclei reacted with *cis*-DDP for the indicated times. Lane 2 and each alternate lane contain the nuclear proteins extracted from untreated nuclei at the indicated times.

reaction and reveals that the intensities of the bands for both the LMG and the HMG proteins 1, 2, and E decrease steadily and at about 5 h are not detectable on the gel. In addition, there were no prominent new bands developing at higher molecular weight (> 24 000), indicating that the HMG proteins were not crosslinking between or among themselves to form higher molecular weight aggregates. The extracted H1 histone also does not appear to be affected under these conditions.

To insure that this finding was not simply a result of *cis*-DDP associated protein degradation, the aliquot taken at 2 h was treated with NaCN to chemically reverse the *cis*-DDP adduct formed. Lanes 1 and 2 in Fig. 12(a) show the level of the proteins in the control and *cis*-DDP treated samples. Lanes 3 and 4 show the same samples after the NaCN treatment. The data show that the reversal treatment, although increasing the extractability of the nuclear proteins, leads to virtually the same intensities for both control and treated samples. This indicates that the proteins were not degraded as a result of the treatment. The *cis*-DDP must therefore react at nucleophilic sites in these proteins, crosslinking them in such a manner that the NHCPs are removed from the gel presentation. This crosslinking could involve either or both protein-protein or DNA-protein crosslinks.

cis-DDP SELECTIVELY CROSSLINKS HMG PROTEINS 1, 2, AND E TO DNA

Proteins that are crosslinked to DNA can be distinguished from those in protein-protein crosslinks by digestion with a variety of nucleases. Figure 12(b) reveals the findings of such a digestion experiment with micrococcal nuclease (M.N.). The 2-h sample was digested to various extents, with Fig. 12(b) showing the results after only 20% acid solubility.

A number of important conclusions are derived from this experiment. First, the band intensities of the HMG proteins 1, 2, and E from the *cis*-DDP treated sample are now comparable to those in the control. The digestion results were virtually the same at even lower levels of digestion, indicating that these *cis*-DDP adducts are very readily excised from the DNA within bulk chromatin. This is even more noteworthy since *cis*-DDP modification of DNA or chromatin significantly inhibits the overall digestion of the DNA backbone [32]. Second, and in contrast to the HMG findings, the band intensities for the LMG proteins remain virtually unaffected and remain undetectable on the gel. This suggests that the M.N. had no effect on their extractability and that these proteins are crosslinked to other high-molecular

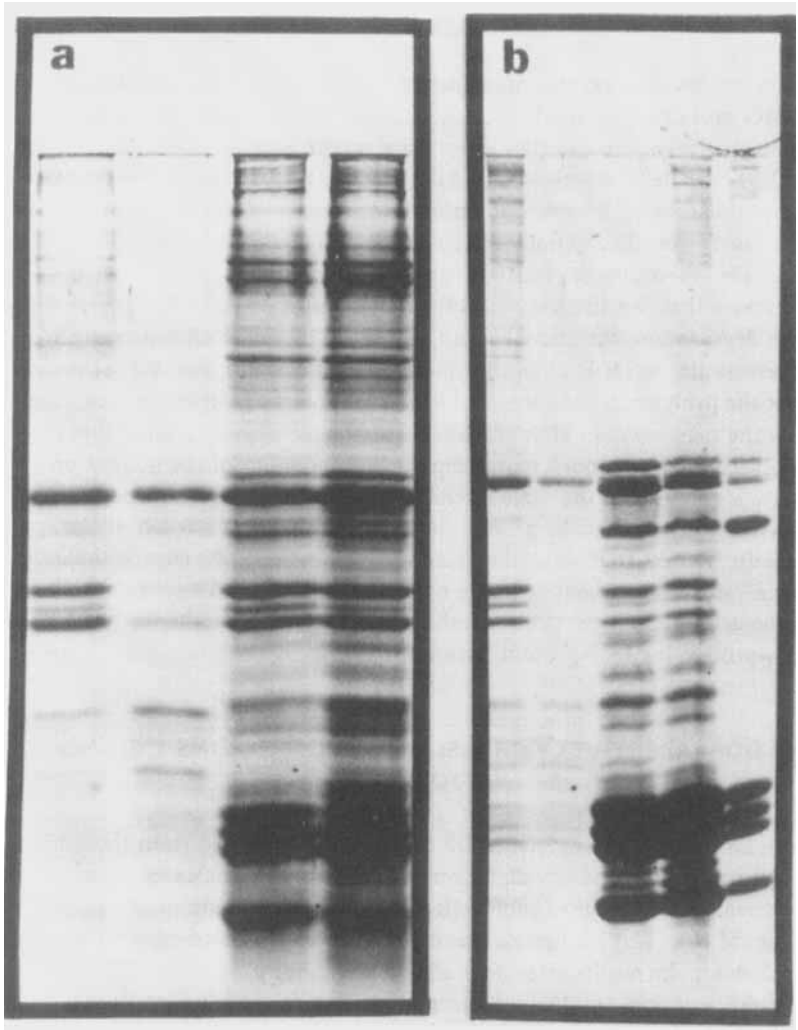


FIG. 12. SDS polyacrylamide gel electrophoresis of extracted nuclear proteins after *cis*-DDP reaction with chicken erythrocyte nuclei and following either (a) NaCN treatment or (b) micrococcal nuclease digestion to 20% acid solubility of DNA. (a) Lanes 1 and 2 show the electrophoretic pattern exhibited by the extracted nuclear proteins after the nuclei, without and with *cis*-DDP, respectively, had been incubated for 2 h under the same conditions as outlined in Fig. 11. Lanes 3 and 4 show nuclear proteins observed after treatment of *cis*-DDP treated and control nuclei, respectively, with 0.2 *M* NaCN for 1 h and then extracted. (b) Lanes 1 and 2 are the same as in (a), while Lanes 3 and 4 show nuclear proteins in *cis*-DDP-treated and control nuclei, respectively, after extraction of micrococcal-nuclease-digested nuclei. Lane 5 contains proteins from (unextracted) nuclei.

weight LMG proteins or perhaps to DNA in regions not accessible to the M.N. Third, the electrophoretic mobilities of the excised proteins are the same as those for the proteins from the untreated control. This indicates that the micrococcal nuclease cuts the DNA in the immediate vicinity of the crosslink, and therefore, few, if any, nucleotides remain attached to the protein.

We have examined this system with other nucleases, including DNase I. We find that this nuclease is far less effective in excising the crosslinked HMGs and, in agreement with the M.N. findings, has no effect on the LMG proteins [33]. DNase I has different characteristics than that of M.N., in that it requires double-stranded DNA and exhibits no preference for the linker region.

MODEL FOR THE INTERACTION OF HMG 1 AND 2 INTERACTING WITH DNA IN CHROMATIN

Although *cis*-DDP is not a zero-length crosslinker, the short crosslinking distance (~3.4 Å) suggests that a segment or domain of these HMG proteins is in the immediate vicinity of, if not in contact with, the DNA. These proteins also must be oriented or positioned relative to one another such that further *cis*-DDP crosslinks do not occur between them or the LMG proteins. Although we cannot eliminate the possibility that the crosslinks originate from dynamically interacting units separated by average distances greater than 3.4 Å, it seems unlikely in light of the extensive level of crosslinking observed.

It has been established that micrococcal nuclease digestion of chromatin exhibits a preference for the linker or internucleosomal region [34]. Since M.N. efficiently cuts out the *cis*-DDP-crosslinked HMG proteins, it strongly supports the notion that these proteins bind directly to the DNA in the linker region in a class of accessible nucleosomal subunits. This work is consistent with and strengthens previous suggestions that HMG proteins 1 and 2 in erythrocytes and mouse myeloma *nucleosomes* [35] and HMG-T in trout testis chromatin [36] reside in the linker region. Importantly, however, our findings pertain to the location of these proteins in *bulk* chromatin, in which the influences of higher order structure are operative. As was noted previously, it is curious that these *cis*-DDP-crosslinked proteins are so readily excised since overall M.N. digestion is significantly inhibited by *cis*-DDP-treated DNA and chromatin [32]. It may be that the nucleosomes which contain these HMG proteins are more accessible to both *cis*-DDP and M.N. It has been suggested that the replacement of H1 histone with HMG 1 and 2 on nucleosomes may be involved in unfolding the local higher-order structure and increasing the accessibility of functional chromatin [35]. In addition, other accounts indi-

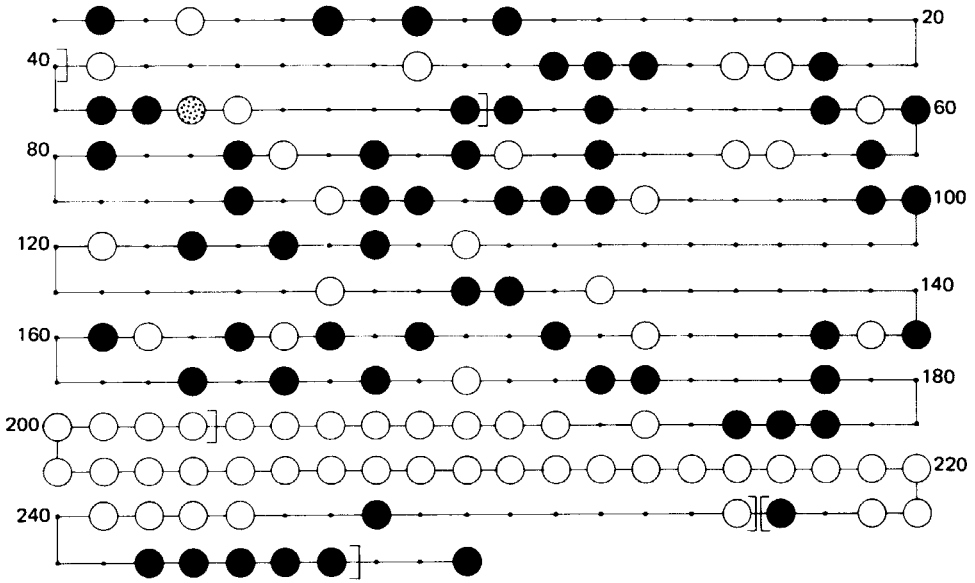


FIG. 13. The distribution of basic (filled circles) and acidic (open circles) amino acid residue in the primary structure of HMG 1 and HMG 2 proteins. The approximate position of cysteine is shown stippled. Regions not yet sequenced are enclosed in parentheses.

cate that HMG 1 binding to DNA [37] and HMG 1 and 2 binding in chromatin [38] increase the rate of M.N. digestion.

A body of evidence suggests that the major site of *cis*-DDP binding is at the N-7 of guanine in DNA [39]. With this being the case, the *cis*-DDP adduct would then reside in the major groove in the B form of DNA and would not of itself produce a significant distortion of the DNA structure [11]. This would also require that the DNA binding domain of the HMGs reside in the major groove. The crosslinking reaction itself and the linkage distance indicate that although the crosslinked partners are in close proximity, they interact loosely enough in native chromatin to permit small molecules such as *cis*-DDP to react with sites on both the DNA and protein.

The HMG proteins 1 and 2 share a great deal of homology and much of the sequence for both has been reported [40]. Figure 13 shows a schematic outlay of the distribution of the acidic and basic residues in HMG 1 and 2, with the general location of one of the four reported cysteines also indicated [41].

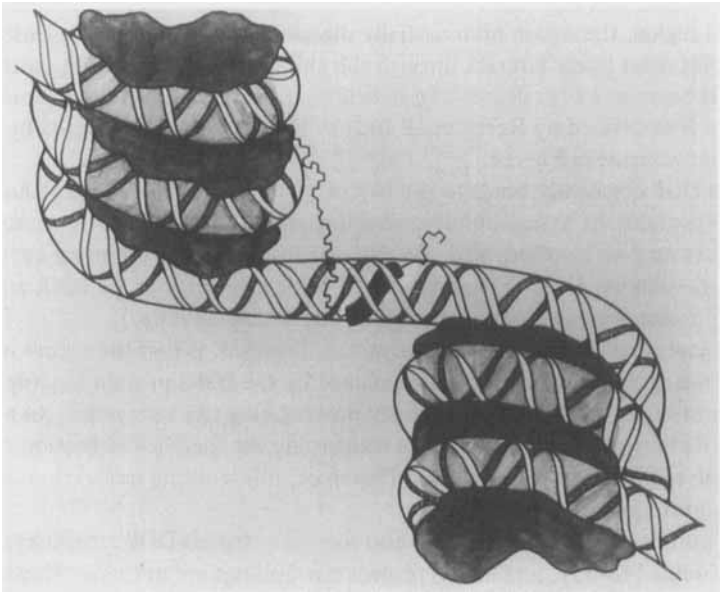


FIG. 14. A dinucleosome segment of chromatin showing the HMG 1 or 2 protein interacting in the major groove of internucleosomal DNA through or immediately adjacent to an α-helical segment. This interaction is most likely associated with the amino-terminal end of the protein in domain A or B [42]. The *cis*-$Pt(NH_3)_2Cl_2$ binds covalently to N-7 of guanine in the major groove and crosslinks to the HMG 1 and 2 via a nucleophile-containing amino acid residue. There is no evidence concerning the specific orientation of the α-helical region of the protein with respect to the DNA and, therefore, the figure should not be overinterpreted.

It has been proposed that the N-terminal and the central domains of these proteins are DNA binding regions and that these domains have substantial α-helical character [42]. Such α-helices have been found to interact in the major groove of DNA in both nonspecific and sequence-specific protein-DNA interactions [43-45].

Collectively, the findings from this study, together with data from previous works [11, 35, 36, 42-47] suggest 1) the following working model for the position of the HMG proteins in bulk chromatin and 2) a description of the *cis*-DDP crosslink between the DNA and these HMG proteins.

Figure 14 shows an idealized picture of a dinucleosome segment of chroma-

tin, with the HMG proteins 1 and 2 interacting with DNA in the internucleosomal region, the region preferentially digested by micrococcal nuclease. The proteins most likely interact through the amino-terminal end of the protein, which contains a high degree of α-helical character (DNA binding domains A and B as defined by Reeck et al. [42]). This region is represented by the tubular element in Fig. 14.

cis-DDP covalently binds to the N-7 of guanine on DNA and crosslinks the HMG proteins via a nucleophile-containing amino acid residue. A number of residues may be involved, with the cysteine residue being a primary candidate. The crosslinking distance is about 3.4 Å. This suggests that the DNA and the HMG proteins are in close proximity or interacting directly.

A caveat regarding this tentative model, however, is that the nature of the local disruption of DNA structure induced by the HMG protein binding itself is yet to be defined. We are currently investigating this very point. In addition, there is currently no evidence concerning the specific orientation of the protein with respect to the DNA. Therefore, this working model should not be overinterpreted.

A number of laboratories have also reported that *cis*-DDP crosslinks DNA to proteins [48-51], and in this respect our findings are not new. However, our lab was the first to *identify* which proteins were crosslinked and subsequently propose a general location for these proteins in bulk chromatin [31]. Knowing the identity of, and with an idea of the general location of these proteins, we can now begin to address an equally important question. That is, what is the function of these proteins in the nucleus, and what influence might the *cis*-DDP crosslinking have on their role in essential cellular metabolism?

At this point, the functional role of the HMG proteins is not clearly defined. However, a number of findings indicate that they may play a necessary role in DNA replication, cellular proliferation, transcription, or other processes prior to terminal differentiation [37, 52-57]. Table 1 lists some of these properties.

In addition, there have been conflicting reports concerned with the role of these proteins in transcriptionally active regions of chromatin [58-60]. These findings open up another area which is worthy of further investigation.

POSSIBLE MECHANISM OF ACTION OF *cis*-DDP IN CHROMATIN

A primary aim of these studies is to interpret our findings in light of the observation that *cis*-DDP selectively inhibits DNA synthesis in mammalian

TABLE 1. Characteristics and Functional Implications of HMG Proteins 1 and 2

1. There are about 10^6 molecules/cell, corresponding to about 1 HMG protein/10 nucleosomes. These proteins are found both in the nucleus and the cytoplasm [52].
2. Both proteins are single-strand DNA-binding proteins [53].
3. Both proteins bind to and unwind nicked circular DNA; HMG protein 2 is twice as effective as HMG 1 [54].
4. a. Levels of HMG protein 2 correlate with the level of proliferative activity in cell types [52].
   b. Level of HMG protein 2α (1/H1°); H1° levels are enriched in nonproliferative cells.
5. Levels of HMG 1 and 2 are about eight times greater in rapidly dividing cultured rat hepatoma cells than in adult rat liver chromatin [37].
6. HMG 1 acts 1) as a helix-destabilizing protein and 2) stimulates in vitro rat-liver DNA polymerase α and β activity. These properties depend on the physiological state of the cell [55].
7. HMG protein 1 acts as a physiological nucleosome assembly factor [56].
8. Levels of HMG proteins 1 and 2 are decreased in cells which have been induced to differentiate [57].

cells, both *in vitro* and *in vivo* [4-6], and, of course, is an effective antitumor drug. The reports from other laboratories on the characteristics of the HMGs which implicate them in DNA replication and cellular proliferation [52-57] permits one now to consider a number of new routes by which *cis*-DDP binding may actually stop cells from dividing. We feel that the clinical efficacy of the antitumor drug may be, in part, associated with the sequestering and "covalently fixing" of these proteins to sites on DNA and therefore restricting their usual participation in aspects of DNA replication and cellular proliferation. Figure 15 shows the replication fork for newly replicating SV40 DNA [61]. As DNA is replicated, the DNA is known to assemble rapidly into nucleosomes and ultimately mold into the higher-order chromatin structure of that cell type. This requires that the population of nucleosomes must

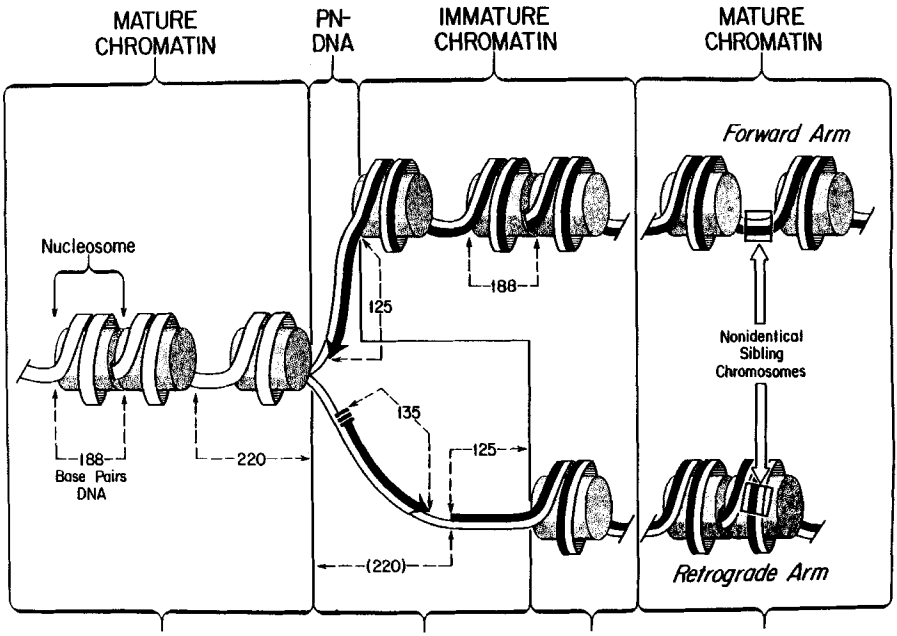


FIG. 15. A model for the replication forks and nucleosome assembly for SV40 minichromosome. Regions include mature chromatin prior to replication, prenucleosomal (PN) DNA at which point nucleosome assembly has not taken place on the DNA (directed downward), immature chromatin, and then the mature chromatin. Numbers refer to approximate sizes of the base pairs involved.

double during each round of the process. If the process of nucleosome assembly does indeed necessitate HMG 1 as a nucleosome assembly factor and HMG 1 and 2 as perhaps single-strand binding proteins, the *cis*-DDP crosslink to the HMG proteins may not permit them to associate with the replication fork area. This may, in a number of ways, inhibit, or perhaps halt DNA replication and thereby inhibit or stop cellular proliferation. In addition, the bulky HMG protein crosslinked to DNA may act to physically block the action of DNA polymerase.

SUMMARY

1. The predominant adduct in DNA, the intrastrand crosslink between adjacent guanines, produces a greater disruption of DNA structure than does the monodentate binding mode.

2. *cis*-DDP prefers to bind to tracts of guanines (in SV40 DNA). In SV40 minichromosome, this would suggest that a primary target would be the topologically exposed, nucleosome-free region, which includes the regulatory region of the genome. The reported inhibition of SV40 DNA synthesis by *cis*-DDP may be directly associated with the disruption of essential sequence-specific protein-DNA interactions.

3. *cis*-DDP preferentially, if not selectively, crosslinks HMG proteins 1, 2, and E to DNA in micrococcal nuclease accessible regions within chromatin. LMG proteins appear to be primarily cross-linked to each other as evidenced by the lack of sensitivity to both micrococcal nuclease and DNase I.

4. A model is suggested for the general location of the HMG proteins 1, 2, and E within chromatin.

5. A plausible, novel mechanism for the antineoplastic action of *cis*-DDP is presented.

ACKNOWLEDGMENTS

This work was supported by Public Service Research Grants from the National Cancer Institute, The Ohio Cancer Research Association, the American Cancer Society, Ohio Division, and FRC and BRSG from Bowling Green State University.

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Note Added in Proof: Subsequent to this meeting, the cDNA for rat liver HMG 1 was reported to contain the complete reading frame coding for the HMG 1 protein [*Nucleic Acids Res., 15,* 9077 (1987)]. The predicted amino acid sequence is similar to that outlined in Fig. 13, but contains 3 cysteine residues.